J. Agric. Sci., Tokyo Univ. Agric., 58 (2), 64–70 (2013)
東京農大農学集報，58（2），64-70（2013）

DNA 1 is no Longer a Constant Feature of the Monopartite Begomovirus DNA β Complex Causing Cotton Leaf Curl Disease in Pakistan

By

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(Received February 19, 2013/Accepted April 19, 2013)

Summary：The association of begomoviruses with single stranded DNA satellites is a relatively recent discovery. Typically the complex consists of a monopartite begomovirus (family *Geminiviridae*) and a small circular satellite, known as a betasatellite that modulates the symptoms induced by the helper virus. Invariably, at least for this complex in Asia, a third molecule, the satellite-like component known as DNA 1, was shown to be present previously in every field. This was certainly the case for the begomovirus betasatellite complex causing cotton leaf curl disease (CLCuD) across Pakistan and western India. By PCR mediated amplification, rolling circle amplification and Southern blot hybridization, we showed that for more recently collected samples, DNA 1 is no longer a constant companion of the CLCuD complex. Sequence analysis of two full-length DNA 1 molecules from 66 infected cotton samples collected in 2005/2006 and 2009/2010 indicated that the few satellite-like components present are typical of the earlier examples. The significance of these findings in relation to recent changes in the CLCuD complex is discussed.

Key words：Begomovirus, *Bemisia tabaci*, Cotton leaf curl disease, Rolling circle amplification

Introduction

A paradigm shift has occurred in the field of plant single stranded DNA (ssDNA) viruses during the last decade. Prior to 1999 it was generally accepted that the majority of begomoviruses (genus *Begomovirus*, family *Geminiviridae*) are bipartite ; their genomes consist of two components, the first (DNA A) encoding all viral factors required for DNA replication, control of gene expression and encapsidation/insect transmission and the second (DNA B) encoding factors required for viral movement in host plants. Certainly in the New World (NW) this remains the case. A monopartite begomovirus, *Tomato yellow leaf curl virus* (TYLCV), occurring in the Caribbean and southern North America, has been introduced from the Old World (OW) during the 1990s (MCGLASHAN *et al.*, 1994 ; POLSTON *et al.*, 1999). Prior to 2000 it is suggested that the majority of begomoviruses occurring in the OW also were bipartite, although a significant number of viruses lacking DNA B component had been identified. The change in our understanding of the genetic make-up of begomoviruses came in 2000 triggered by the identification of satellites of begomoviruses (BRIDDON *et al.*, 2003 ; SAUNDERS *et al.*, 2004). The distribution of virus types now shows the monopartite begomoviruses to outnumber the bipartite viruses, and the majority of the monopartite viruses has an association with the newly identified satellite known as betasatellites.

DNAβ (DNA betasatellites) molecules are symptom modulating ssDNA satellites associated with begomoviruses. DNAβ is approximately half the size of their helper begomoviruses encoding a single gene (βC1) that mediates all identified satellite functions including pathogenicity, overcoming host plant defenses through post transcriptional gene silencing and up regulation of virus DNA levels and possibly is also involved in virus movement in plants (CUI *et al.*, 2005 ; SAEED *et al.*, 2004 ; SAUNDERS *et al.*, 2004 ; SAEED *et al.*, 2007). In case of the begomovirus betasatellite complexes causing cotton leaf curl disease (CLCuD), the βC1 gene has been shown to be the major symptom determinant that is responsible

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for the characteristic symptoms of the disease (Qazi *et al.,* 2007).

A further group of ssDNA molecules, known as DNA 1 was shown to be associated with begomoviruses that require a DNA betasatellite. In case of begomoviruses of the Indian subcontinent, all begomovirus betasatellite infected plants were shown also to contain this molecule. Only some viruses originating from the Far East, like *Honeysuckle yellow vein virus* (HYVV) and *Eupatorium yellow vein virus* (EuYVV) are not associated with DNA 1 molecules (Briddon *et al.,* 2004). DNA 1 molecules are satellite like, circular, ssDNA components approx. half the size of their helper begomoviruses and encode a single product ; a rolling circle replication initiator protein (the replication associated protein [Rep]) (Briddon *et al.,* 2003). Interestingly DNA 1 molecules are closely related to the Rep encoding components of a second family of ssDNA viruses, the nanoviruses, from which they are believed to have evolved following component capture (Mansoor *et al.,* 1999 ; Saunders *et al.,* 1999).

CLCuD is caused by a begomovirus betasatellite complex consisting of multiple distinct begomoviruses supported by a single type of betasatellite (Mansoor *et al.,* 2003). The disease first appeared in the mid-1980s and was epidemic during the 1990s causing major losses to cotton production across Pakistan and western India. The introduction of resistant cotton varieties in the late 1990s restored production levels to their pre-epidemic levels. However, in 2002 a resistance breaking strain of the disease appeared and rapidly spread (Mansoor *et al.,* 2003 ; Mansoor *et al.,* 2006). At this time there are only tolerant cotton varieties available to farmers and losses are again high (Rahman *et al.,* 2005). The objective of this study was to isolate, identify and characterize DNA 1 from the cotton leaf curl disease complex in Punjab and Sindh provinces of Pakistan.

Materials and Methods

Sample collection and DNA extraction

A total of 66 leaf samples originating from Sindh and Punjab provinces, Pakistan, was collected in 2005/2006 and 2009/2010 from cotton plants with unambiguous symptoms of CLCuD. Nucleic acids were extracted from frozen leaf samples by the CTAB method (Doyle and Doyle, 1990). Total nucleic acid extracts were suspended in sterile distilled water and maintained at −20℃. All the experiments were conducted in Pakistan and only further analysis was conducted at Tokyo University of Agriculture, Japan.

PCR amplification and cloning

To assess the presence of DNA 1, DNA101/DNA102 universal primers were used to amplify the full-length begomovirus DNA 1 component (Bull *et al.,* 2003). The expected sizes of amplification products of DNA 1 were cloned into the pTZ57R/T using the InsT/Aclone kit (Fermentas, Vilnius, Lituania). The amplified product of expected size was cloned and sequenced entirely in both orientations using primer walking strategy commercially (Macrogen, Inc., Seoul, South Korea).

Southern hybridization

Total genomic DNA was isolated from cotton plants. Total nucleic acid (10 μg) was resolved in 1.5% agarose gel and run at 40 V (Biorad PowerPac^{TM}) in TBE buffer for 4 to 5 h. Gel was stained with 0.5 μg/mL ethidium bromide and DNA image was obtained under UV light in gel documentation apparatus (Eagle Eye-Stratagene). After electrophoresis the gel was treated with depurination solution (0.25M HCl) for 15 min, denaturation solution (1.5M NaCl and 0.5M NaOH) for 30 min and neutralization solution (1M Tris [pH 7.4], 1.5M NaCl) for 30 min. The gel was rinsed briefly with distilled water between treatments and shaken moderately on platform shaker during each treatment. DNA in the gel was transferred to a nylon membrane(Hybond-Amersham Pharmacia Biotech, NJ, USA) in 10X SSC and sometimes in 5X SSC (1.5M NaCl and 150 mM sodium citrate) by capillary action. The DNA on the nylon membrane was crosslinked by UV irradiation (CL-1000 , UVP) at 120 mJ/cm^2 energy. The membrane was then rinsed in a solution containing 0.1X SSC, 0.5% (w/v) SDS at 65℃ for 45 min to remove residual agarose and transferred to nylon membranes (Hybond XL-Amersham Pharmacia. Biotech, NJ, USA). Before hybridization the membrane was treated with 0.2 ml/cm^2 pre-hybridization solution (6X SSC, 5X Denhardt's solution [0.1% (w/v) each of bovine serum albumin, 0.5% (w/v) Ficoll (Mol. Wt. ～400,000) and PVP (Mol. Wt. ～40,000)], 50% (v/v) SDS) and 5 mg/mL sheared and denatured salmon sperm DNA at 42℃ for 2–4 h in a hybridizer (Hybaid, Midi Dual 14), to block non-specific binding sites. DNA 1 probes were prepared using a Biotin DecaLabel DNA Labelling kit (Fermentas, Vilnius, Lituania) according to the manufacturer's instructions. Briefly, in a 1.5 mL microcentrifuge tube a 44 μL reaction mixture was prepared by adding 50–200 ng DNA template (usually purified PCR product), 10 μL decanucleotide in 5X reaction buffer and nuclease free water. The reaction mixture was vortexed briefly, centrifuged briefly in a microfuge to collect the contents at the bottom of the tube and incubated in a boiling water bath for 5–10 min. After incubation the tube was cooled on ice, briefly microfuged and the contents of the tube were mixed with 5 μL biotin labelling mixture and 1 μL Klenow fragment

exo- (5 units) and incubated at 37℃ for 1 to 20 h. Reaction was stopped by adding 1 μL 0.5M EDTA [pH 8.0]. To prepare hybridization solution, the biotin labelled probe was denatured at 100℃ for 5 min, chilled on ice and mixed with pre-hybridization solution (25–100 ng/mL).

After 2–4 hrs treatment, the pre-hybridization solution was discarded and the hybridization solution was added to the membrane (60 μL/cm^2) and incubated overnight in a hybridizer at 42℃. The following day the membrane was washed twice with 2X SSC/0.1% (w/v) SDS at room temperature for 10 min. The membrane was washed with 0.1X SSC/0.1% (w/v) SDS twice at 65℃ for 20 min. To detect the biotin-labelled DNA the membrane was washed in 30 mL Blocking/Washing Buffer (provided by the manufacturer) at room temperature. After 5 min the membrane was treated with 30 mL Blocking Solution for 30 min to block non- specific binding sites on the membrane. Streptavidin-AP conjugate was diluted in 20 mL Blocking Solution and the membrane was incubated in it for 30 min. The membrane was washed twice in 60 mL Blocking/Washing buffer for 15 min and incubated with 20 mL Detection Buffer for 10 min. Finally the membrane was treated with 10 mL freshly prepared Substrate Solution at room temperature in the dark until blue-purple precipitate became visible. To stop the reaction, the substrate solution was discarded and the membrane was rinsed with water. The blot was immediately photographed (Fig. 1) and the membrane was then air dried and stored.

Rolling circle amplification

To account for the possibility that DNA 1 are present in many plants but at levels below the detection threshold of PCR and Southern blot hybridization, we employed rolling circle amplification (RCA) using Φ29 polymerase (Fermentas, Vilnius, Lituania) to initially amplify all circular DNA molecules present in 10 DNA extracts from CLCuD affected plants from Punjab and Sindh provinces of Pakistan (HAIBLE *et al.,* 2006). A high molecular weight product was produced in all RCA reactions. Equal amounts of the amplification product were then digested with the restriction endonuclease *Pst*I, run on an agarose gel, transferred to nitrocellulose and probed by Southern hybridization for the presence of DNA 1. Old DNA 1 probe (accession no. AJ132344) was used for radioactively labeling.

Sequencing

Two clones, shown to contain a 1.4 kb insert upon digestion with specific restriction enzyme were selected for further analysis. The plasmid DNA was purified using a GeneJET Plasmid Miniprep Kit (Fermentas, Vilnius, Lituania) and sequenced commercially (Macrogen, Inc., Seoul, South Korea). Sequence information was assembled using the SeqMan program of the Lasergene sequence analysis package (DNAStar Inc., WI, USA). ORFs were predicted using ORF Finder run online (NCBI). Sequence alignments and phylogenetic trees were constructed using the neighbour joining algorithm of CLUSTAL X (THOMPSON *et al.,* 1997). Phylogenetic dendrograms were viewed, manipulated and printed using Treeview (PAGE, 1996).

Results and Discussion

Detection of DNA 1component by Southern hybridization

DNA 1 was detected from 2 samples by PCR out of 66 cotton samples. They were cloned into the cloning vector and were further confirmed by Southern hybridization using RCA amplified genomic DNA. Both DNA 1 have an arrangement that is typical of previously characterized DNA 1, containing a single ORF in the virion-sense similar to the replication-associated protein (Rep ; a rolling circle replication initiator protein of nanoviruses, an A-rich sequence (with 49% adenine content) and a predicted hairpin structure with the loop sequence TAGTATTAC typical of DNA 1.

Analysis of sequences

The complete sequences of two clones were determined. These sequences are available in the databases under accession numbers AM711115 and AM711116, for the 2005 and 2006 samples respectively. The sequences of the DNA 1 are 1380 and 1374 nucleotides in length, for CLCuD DNA1-[PK:Shad:05] and CLCuD DNA1-[PK:Shad:06], respectively. They have the typical arrangement of DNA 1 molecules described above with similarity to the origin of virion strand DNA replication of the geminiviruses and nanoviruses (BRIDDON *et al.,* 2004 ; MANSOOR *et al.,* 1999 ; SAUNDERS and STANLEY 1999). The stem sequences of the two molecules are distinct, being GCTCCGCCC and TGGCTCCGACC for the 2005 and 2006 isolates, respectively. Nevertheless, they fall into the groups of stem sequences (groups 1 and 2, respectively) shown previously to be present in DNA 1 molecules identified from CLCuD affected cotton on the subcontinent (BRIDDON *et al.,* 2004). Sequence comparisons with all available DNA 1s in the databases showed that first DNA 1 has the highest nucleotide sequence identity (82.8%) with a DNA 1 isolated from tomato leaf curl disease affected tomato in Pakistan (accession no. AJ 512955), while the second showed the highest levels of nucleotide sequence identity (79.2%) to a DNA 1 isolated

from CLCuD affected cotton (AJ132344), the first such molecule to be identified (Mansoor *et al.*, 1999) (Table 1).

Phylogenetic analysis

A phylogenetic analysis, based upon an alignment of the complete nucleotide sequences of the majority of previously characterized DNA 1 molecules, is shown in Fig. 2.

This shows the two recently isolated CLCuD associated DNA 1 molecules to segregate with DNA 1s previously identified in association with the disease ; being most closely related to a DNA 1 isolated from tomato leaf curl disease (ToLCD) affected tomato (AJ512955) and a DNA 1 isolated from CLCuD affected cotton (AJ132344).

The precise function of DNA 1 in begomovirus DNA β infections is not clear yet, although recent work has suggested some possibilities. The component is not required for infectivity or symptom induction (Mansoor *et al.*, 1999 ; Saunders and Stanley 1999, Wu and Zhou 2005). Recent studies have shown that the A-rich region of DNA 1 does not effect its replication (Shahid *et al*., 2009). Nevertheless, in previous analyses DNA 1 was invariably found associated with CLCuD and a number of other begomovirus DNA β complexes. This is suggestive of a useful, if not absolutely essential, contribution of the satellite like molecule to begomovirus DNA β complexes. Upon their first identification it was suggested that the function of DNA 1 may be to down regulate the severity of the symptoms caused by the complex, by mopping up cellular resources (Mansoor *et al.*, 2000 ; Saunders and Stanley 1999). Wu and Zhou (2005) demonstrated that the presence of DNA 1 reduced virus DNA levels, but only in the absence of DNA β, although symptoms in both cases were attenuated at the early stages of infection. If DNA 1 acts as a "dampener" of the virus infections, then we must assume that its dampening effect is no longer a requirement of the CLCuD complex presently in the fields in Pakistan.

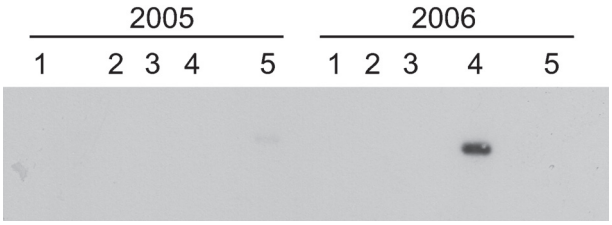

Fig. 1 Southern blot of DNA extracts from CLCuD affected cotton plants probed with a radioactively labeled DNA 1 probe (accession no. AJ132344). Approx. 10 μg of DNA was loaded in each lane with the exception of lane 1 which was loaded with approx. 5 ng of an undigested clone of DNA 1 (accession no. AJ132344) as a hybridization control.

The CLCuD complex has gone through a major change since the DNA 1 molecules were first identified. The previous strain of the disease, now known as the "Multan strain", has been almost entirely displaced by the "Burewala strain". This new strain is resistance breaking and able to infect cotton varieties derived from the germplasm LRA5166 and CP15/2 with resistance to the Multan strain (Rahman *et al.*, 2005). The resurgence has again led to major losses due to the disease in Pakistan and the Burewala strain is now also present in western India. We have previously shown that the Burewala strain is associated with a recombinant DNA β, for the most part, consisting of sequences derived from the Multan strain betasatellite but with a small region of sequence within the satellite conserved region (SCR) derived from a ToLCD associated betasatellite (Amin *et al.*, 2006 ; Briddon *et al.*, 2003). The significance of this recombination between cotton and tomato betasatellites remains unclear due to the fact that we have yet to understand the precise function of the SCR. More recently the begomovirus associated with the Burewala strain has been sequenced and also shown to be recombinant (Amrao *et al.*, 2010).

The presence of DNA 1 in only a small number of plants indicates that, in contrast to earlier analyses, DNA 1 is no longer a constant feature of the CLCuD begomovirus betasatellite complex. It is likely that the changes in the

Table 1 The highest and the lowest percentage nucleotide sequence identities of the DNA1 isolated from cotton with all available sequences of other DNA1 in the databases

[PK:DSB:05]	[PK:DSC:06]	YLCCNA(16)*	TbCSA(8)*	AYVA(6)*	HLCuA(4)*	ToLCA(5)*	CLCuMA(4)*	MalYMA(5)*	SiLCuA(4)*	MimLCuA(1)*	OLCuA(1)*	OKLCuA(1)*	
100	67.4	64.6-68.4	66.9-69.2	62.3-70.4	74.4-78.8	75.5-82.8	69.4-71.4	66.4-65.5	63.9-68.1	67.0	76.0	65.3	[PK:DSB:05]
	100	65.1-70.4	68.6-71.3	61.4-76.1	68.1-69.7	67.8-70.3	75.7-80.1	75.7-79.1	65.5-78.1	72.4	78.7	64.0	[PK:DSC:06]
		100	77.4-81.3	60-73.9	67.2-68.7	67.5-70.8	63.9-71.6	63-73	66.6-69.4	61.5-78.2	64.8-69.1	63.3-68.1	TYLCCNA
			100	62.8-76.4	68.6-71.1	70.5-73.1	66.8-71.6	69.8-71.9	67.2-70.5	69.1-82.7	66.2-69.5	63.5-68.6	TbCSA
				100	79.6-81.3	69.4-80.4	67.2-76.9	72.4-72.6	68.3-74.5	65.5-72.4	61.1-68.8	63.2-65.9	AYVA
					100	74-86.1	66.8-72.2	67.2-68.4	64.7-70.7	66.5-70.4	65.2-77.8	65.3-71.5	HLCuA
						100	67.4-72.6	65.5-68.8	65.4-70.8	71.5-61.9	68-73	64.3-68.9	ToLCA
							100	66.7-98.2	63.3-77.6	68.4-69.6	68.1-71.5	62.3-70.5	CLCuMA
								100	66.5-80.1	64.2-74.5	69-81.1	62.3-68.5	MalYMA
									100	69.0	61.5-64.2	61-63.1	SiLCuA
										100	64.4-84.8	62.5	MimLCuA
											100	62.5	OLCuA
												100	OKLCuA

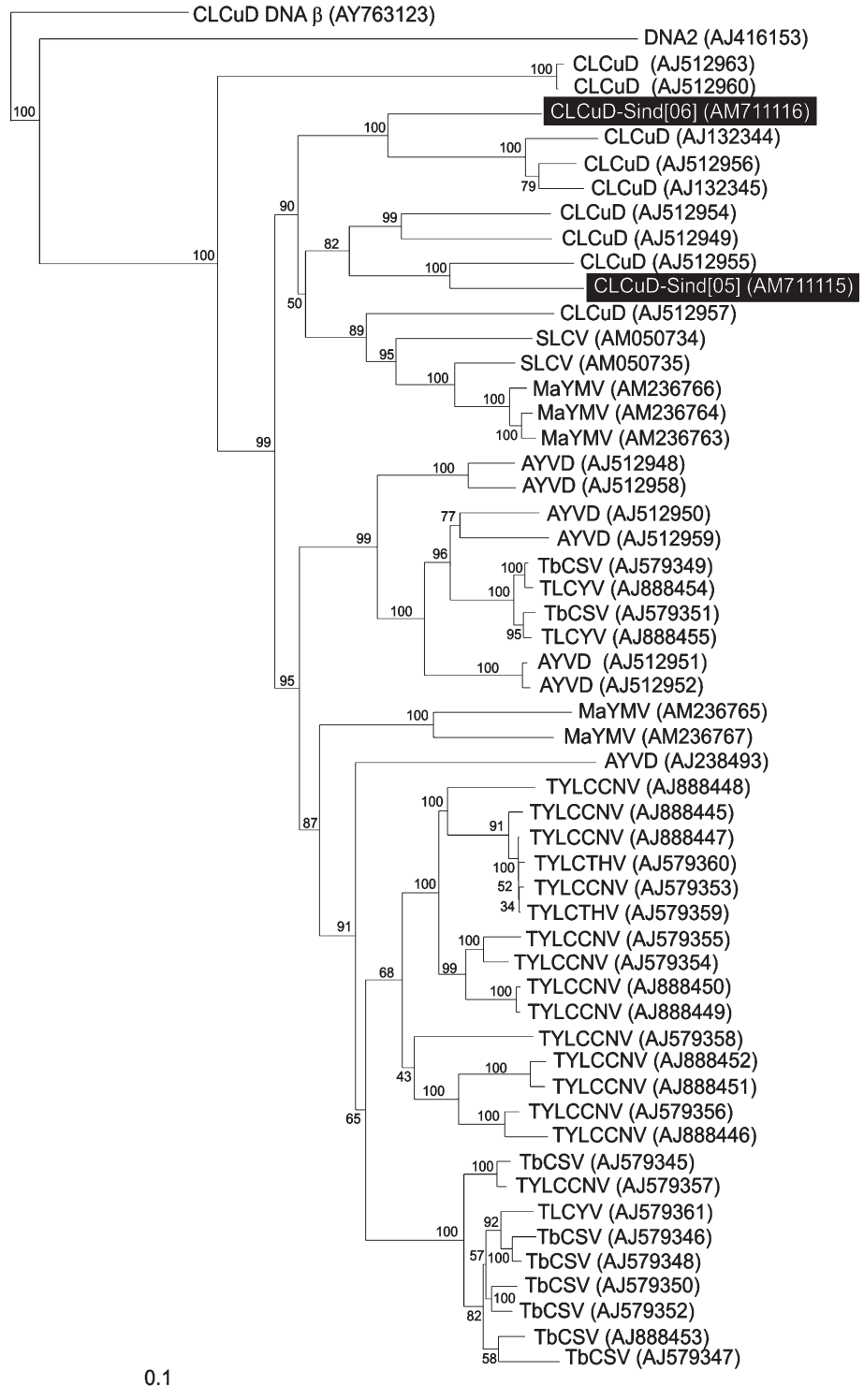

Fig. 2 Neighbour-joining phylogenetic dendrogram based upon an alignment of the complete nucleotide sequences of the majority of DNA 1 sequences available in the databases. Numbers at nodes indicate percentage bootstrap scores (1000 replicates). The database accession numbers of each DNA 1 are given in brackets. The DNA 1 is additionally labelled by the virus with which they are associated (where known) or the disease with which they are associated. The abbreviations used are *Ageratum yellow vein virus* (AYVV), *Squash leaf curl virus* (SLCV), *Malvastrum yellow mosaic virus* (MYVMV), *Tobacco curly shoot virus* (TbCSV), *Tomato yellow leaf curl China virus* (TYLCCNV), *Tomato yellow leaf curl virus* (TYLCV).

CLCuD complex that we have detailed here are also occurring in other begomovirus betasatellite complexes. Similarly a recent analysis of the ToLCD complex in Pakistan has been unable to consistently detect the DNA 1 component. The significance of these findings are unclear. What it does indicate is that the situation with the begomovirus/DNA β complexes is far from stable, with major changes taking place in the prevalent viruses and the make-up of the constituent components of the complexes, particularly for CLCuD.

Acknowledgements

The first author would like to thank "Higher Education Commission" (HEC) of Pakistan for providing funds and National Institute for Biotechnology and Genetic Engineering (NIBGE) for producing the constructs. The authors also thank NODAI Research Institute (NRI), Tokyo University of Agriculture, Japan for the Grant in Aid in Advanced Research Project for further analysis.

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パキスタンの cotton leaf curl disease の病原である単一ゲノムタイプのベゴモウイルスと DNA β サテライト複合体において DNA 1 の随伴は常には起きない

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（平成 25 年 2 月 19 日受付/平成 25 年 4 月 19 日受理）

要約：ベゴモウイルスに 1 本鎖 DNA サテライトが付随することは比較的最近の発見であるが，これらの複合体は単一ゲノムタイプのベゴモウイルス（ジェミニウイルス科）とベータサテライトと呼ばれる小さい環状サテライトからなり，サテライトはヘルパーウイルスの病徴に関与する。アジアではこの複合体に，さらに 3 番目の DNA 1 として知られるサテライト様粒子が常に付随していることが示されており，パキスタンとインド西部に広まっている cotton leaf curl disease（CLCuD）の病原も，このような複合体からなっているとされてきた。しかし，本研究において PCR 法による増殖，ローリングサークル法による増殖およびサザンブロットハイブリダイゼーションにより，近年採集した試料では DNA 1 は必ずしも CLCuD の病原複合体に付随しないことを認めた。すなわち，2005/2006 採集および 2009/2010 採集の CLCuD 罹病ワタ 66 試料からは 2005/2006 採集の 2 試料のみが DNA 1 を付随しており，その全塩基配列を解析し，DNA 1 が付随しない CLCuD 病原の発見について議論した。

キーワード：ベゴモウイルス属，*Bemisia tabaci*，Cotton leaf curl 病，ローリングサークルアンプリフィケーション

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